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13. ABSTRACT *(Maximum 200 Words)*

Several humans displayed marked similarity in the rates and properties of chromium reduction catalyzed by hepatic microsomal enzymes. Kinetic parameters demonstrated significant rates of chromium reduction using chromium(VI) concentrations anticipated for occupational exposure, and the reactive intermediate chromium(V) was formed. Room air inhibited only a minority of total chromium(VI) reduction.

Iron, at concentrations well below that of chromium(VI), markedly stimulated the rates and V_{max} of chromium(VI) reduction in both liver and lung, but did not significantly change other parameters. The iron effect was not altered by O_2. Individuals who are simultaneously exposed to chromium and iron, or other compounds that mediate iron release from ferritin, are likely at greater risk for chromium toxicity.

Cytochromes P450 and flavin-containing monooxygenase (FMO3) were not capable of mediating chromium(VI) or iron(III) reduction. P450 reductase was a poor mediator of reduction on its own, but played a significant participatory role in chromium(VI) reduction. Cytochrome b_5 significantly stimulated rates of chromium(VI) reduction, probably by acting in cooperation with P450 reductase and b_5 reductase.

All findings showed significant differences from exisiting rodent models, emphasizing the need to utilize human studies to understand chromium toxicity in humans.

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Final Technical Report (Cumulative for 1 April 1995 – 31 March 1998)
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CHROMIUM TOXICITY: REDUCTIVE ENZYMES IN HUMANS
Principal Investigator: Charles R. Myers, Ph.D., Medical College of Wisconsin
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ORIGINAL OBJECTIVES:

Exposure to chromium (Cr) compounds is associated with a wide array of toxic effects, including increased incidence of certain cancers [3,12,27,29,64], serious damage to internal organs (e.g. lungs, liver, kidneys) [24,27,64], pulmonary fibrosis and chronic bronchitis [2], skin ulcers and allergic dermatitis [2], and impairment of primary immune responses [17]. The inhalation of Cr-containing fumes, dusts, and particles is primarily associated with respiratory tract damage [12,22,61], whereas exposure to forms which are internalized is associated with toxicity to other organs including liver and kidneys [25,65].

The solubility and membrane permeability of various Cr compounds likely play key roles in Cr toxicity/carcinogenicity: Cr(III) compounds are generally quite insoluble and, as such, do not easily cross cell membranes [26]; in contrast, Cr(VI) compounds, many of which are soluble, readily cross the skin [2] and are readily transported across cell membranes [18] via an anion carrier [6]. Cr compounds are also mutagenic [67], and the bulk of *in vitro* studies have implicated exposure to Cr(VI) as the predisposing factor to Cr-induced genotoxicity [7,11,30,66]. However, Cr(VI) is not itself likely the toxic species. Rather, the reduction of Cr(VI) to Cr(III), and the resulting formation of reactive intermediates Cr(V) and/or Cr(IV), seems to be a key component in the cytotoxicity and genotoxicity of Cr(VI) compounds [28,60,66].

The molecular mechanisms of the genotoxic and cytotoxic effects of Cr(VI) are, at best, only partially understood, and most studies have utilized rodents or purified reducing agents to elucidate potential reductive processes and their implications. However, in a preliminary report using hepatic tissue from primarily one human, we noted certain key differences in microsomal Cr(VI) reduction between rodents and humans [53]. A thorough understanding of the mechanisms of Cr(VI) reduction in humans is therefore necessary, especially since the levels and nature of the reductants can significantly influence the levels and types of reactive intermediates generated.

Therefore, to gain further insight into the mechanisms of chromium(VI) reduction in humans, the original specific aims of this project were to:

(1) determine the general characteristics of chromium(VI) reduction by human hepatic microsomal enzymes, including determination of both the kinetic aspects of, and interindividual differences in, microsomal chromium(VI) reduction;
(2) elucidate the effects of various levels of O_2 on human hepatic microsomal chromium(VI) reduction;
(3) examine the potential irreversible binding of chromium to microsomes as a result of chromium(VI) reduction, and the potential effects of this binding on microsomal enzyme activities; and
(4) determine the ability of key microsomal enzymes, including NADPH:cytochrome P450 reductase and flavin-containing monooxygenase(s), to mediate chromium(VI) reduction.

OVERVIEW OF OBJECTIVES ACCOMPLISHED:

Aims #1 and #2 have been completed as planned. Human hepatic microsomal enzymes can mediate chromium(VI) reduction in an NADPH-dependent manner at significant rates using chromium(VI) concentrations well below those anticipated for occupational exposure. There was little difference in chromium-reducing rates or properties when comparing all five individuals

examined, with an apparent K_m for chromate of 1.04–1.68 μM, and a V_{max} of 10.4–10.7 nmol min^{-1} mg $protein^{-1}$. Relative to anaerobic conditions, Cr(VI) reduction was inhibited only 26–37% by room air, which indicates that human microsomal Cr(VI) reduction could still proceed at significant rates even in tissues with high O_2 tensions. Inhibitor studies indicate no role for cytochrome P450s, but a prominent role for flavoproteins. While the emphasis of the project was on Cr(VI) reduction by hepatic microsomes, studies with lung microsomes from one human exhibited V_{max} and K_m values that were two-thirds lower and 2.8-fold greater, respectively, than those of hepatic microsomes from the same individual; other Cr(VI)-reducing parameters were similar for lung and liver.

Significant progress has been made toward accomplishing aim #4. We have discovered that the enzymatic chromium-reducing mechanisms in humans are more complex, and somewhat different, than originally perceived. Contrary to preliminary inhibitor data, the use of a purified recombinant flavin-containing monooxygenase isoform 3 (FMO3) suggests that FMO3 does not have a prominent role in Cr(VI) or Fe(III) reduction. Consistent with inhibitor data, human cytochrome P450 reductase plays a significant role in microsomal Cr(VI) reduction, but by itself is a relatively poor reducer of Cr(VI) and Fe(III). It apparently acts in concert with other microsomal components, e.g. cytochrome b_5, to mediate significant rates of Cr(VI) reduction. While not among our original aims, we have discovered that cytochrome b_5 reductase does not appear to directly mediate Cr(VI) or Fe(III) reduction at significant rates, but could play a significant role by acting in concert with cytochrome b_5. The various tools (e.g. antibodies, recombinant proteins) are now in hand to further pursue details of the interaction of cytochrome b_5 with P450 reductase and b_5 reductase in the reduction of chromium(VI).

While not included in the original aims, it is now apparent that human hepatic microsomes generate the reactive intermediate Cr(V) during the reduction of Cr(VI). Since Cr(V) is known to lead directly to the generation of reactive oxygen species and the formation of lipid hydroperoxide radicals from previously existing lipid peroxides, this highlights the potential toxicologic consequences of microsomal Cr(VI) reduction.

Furthermore, a strong link between the chromium and iron reductase activities of human microsomes was identified. Specifically, levels of iron that are 3.1- to 26-fold lower than the initial Cr(VI) concentration markedly stimulated microsomal Cr(VI) reduction rates, consistent with a repeated redox-cycling of a small amount of iron as a means to mediate the reduction of a much larger amount of Cr(VI). The concentration of iron markedly influenced the rate and V_{max} of Cr(VI) reduction, whereas other Cr(VI)-reducing parameters were only influenced to a small extent by iron. The effect of iron is independent of the presence or absence of O_2, and the relative effect of iron on Cr(VI) reduction rates is similar for lung and liver microsomes. Hence, individuals who are exposed to exogenous iron or to compounds that can mediate increases in intracellular iron, are likely to be at greater risk for the toxicologic consequences associated with intracellular Cr(VI) reduction.

The unexpected discovery of a significant role for iron also invalidates much of the original reasoning for pursuit of aim #3. The idea was that an enzyme which directly mediated Cr(VI) reduction would likely be subject to preferential free radical attack by reactive chromium intermediates, because of its immediate proximity to these intermediates as they are formed. Since much of the microsomal Cr(VI)-reducing activity now seems to be mediated indirectly via the redox cycling of iron, such preferential attack now seems unlikely. For this reason, aim #3 was not pursued.

The sum total of findings support the original contention that the rodent model is not appropriate to understand the generation of reactive chromium intermediates catalyzed by human microsomal enzymes. Continuation with these human studies is therefore necessary to understand chromium(VI)-mediated toxicity and carcinogenicity in man.

ACCOMPLISHMENTS, NEW FINDINGS, AND THEIR IMPLICATIONS:

Aims #1 and #2, Accomplishments

We have completed a thorough analysis of various parameters of Cr(VI) reduction catalyzed by hepatic microsomes from five individuals. The findings, which are summarized in Table I, show no major interindividual differences, suggesting that the responsible components are present at similar levels in different individuals. In all cases, Cr(VI) reduction was enzymatic and NADPH-dependent, and rates were proportional to the amount of microsomes added. Of special note, and in marked contrast to rodent studies, are the very low K_m for Cr(VI) (1.04 to 1.68 μM), and the lack of effect of any of the cytochrome P450 inhibitors (Table I)[46]. These results are not likely to be a function of unusual or low P450 levels in these individuals, since published data [9,73] indicate that they represent a broad range of levels of various P450 isoforms. For hepatic microsomes, total cytochrome P450 contents ranged from 0.16 to 0.33 nmol P450 mg protein^{-1}, and P450 reductase activities ranged from 73 to 110 nmol cytochrome *c* reduced min^{-1} mg protein^{-1}. Cytochrome b_5 contents were 0.40, 0.39, and 0.41 nmol/mg protein for humans C, N, and O, respectively. Given the pronounced similarity in Cr(VI)-reducing properties between these five individuals, these studies were not extended to more individuals.

While the primary focus of the project was to examine Cr(VI) reduction by human hepatic microsomes, we had the opportunity to examine Cr(VI) reduction using lung microsomes from patient O, on which we also had hepatic data. The V_{max} for Cr(VI) reduction observed with these lung microsomes (3.6 ± 0.7 nmol/min/mg) was approximately one-third of the hepatic V_{max} in this patient. The apparent K_m for Cr(VI) for these lung microsomes was 4.6 μM, which is 2.8-fold greater than that of liver microsomes in the same patient [46]. Similar to the liver, various cytochrome P450 inhibitors did not affect Cr(VI) reduction by lung microsomes [46]. For these lung microsomes, total cytochrome P450 content was below detectable limits and P450 reductase activity was 37 nmol cytochrome *c* reduced min^{-1} mg protein^{-1}. Similar to liver, lung microsomal Cr(VI) reduction was stopped by heat-denaturing the microsomes or by excluding NADPH (not shown). Also similar to the liver, $TlCl_3$ markedly inhibited lung microsomal Cr(VI) reduction suggesting a role for flavoproteins [46].

These results demonstrate that the rates and basic properties of human microsomal Cr(VI) reduction are very similar in different individuals (Table I). As in rodents [39], human microsomal Cr(VI) reduction is NADPH-dependent. The V_{max} in human hepatic microsomes (10.4–10.7 nmol min^{-1} mg protein^{-1}) is somewhat greater than that reported for control rats (6.4 ± 0.1 nmol min^{-1} mg protein^{-1}) [39]. However, with respect to other key properties, the microsomal Cr(VI)-reducing system in humans is very distinct from that in rats. The apparent K_m for chromate of the human system (1–1.7 μM) is three orders of magnitude lower than that reported for rat liver microsomes (1.6 mM) [16]. This implies that humans could be more susceptible to Cr(VI)-reduction-linked cellular damage as the microsomal Cr(VI)-reducing enzymes would likely be active at Cr(VI) concentrations that are reasonable for occupational or environmental exposure. Similarly, the apparent K_m for Cr(VI) for lung microsomes (4.6 μM) is well within the realm of occupational exposure and is consistent with a potentially prominent role for microsomal enzymes in the lung. It must be understood that there are many different cell types in the lung, so studies with lung microsomes only provide a conglomerate of the Cr(VI) reduction processes in various cell types. Since the suspect Cr(VI)-reducing enzymes (see below) are expressed at widely different levels among various lung cell types, different components may be responsible for Cr(VI) reduction in different lung cell types; in addition, certain lung cell types could display significantly different rates of Cr(VI) reduction. Nonetheless, the general properties of microsomal Cr(VI) reduction in human lung do not appear to be markedly different from those in liver from the same patient, and are consistent with a potentially significant role for microsomal enzymes.

In marked contrast to rodents, in which cytochrome P450(s) can mediate Cr(VI) reduction [38-40], none of the human microsomes displayed any sensitivity to various cytochrome P450 inhibitors (Table I)[46]. However, marked inhibition by $TlCl_3$ suggests a prominent role for

flavoproteins in humans; the effects of $TlCl_3$ could be due to its potential action as a flavoprotein antagonist and/or as an inhibitor of the active sulfhydryl groups of P450 reductase [71]. The inhibitory effect of BNAP (Table I), which inhibited >90% of the P450 reductase activity (not shown), further implicates a role for P450 reductase [21]; while BNAP can also inhibit certain cytochrome P450s [52], the lack of effect of other P450 inhibitors (Table I) suggests that the effects of BNAP are due to its ability to inhibit P450 reductase. While purified rat P450 reductase can, when present at high levels, reduce Cr(VI), this activity is very O_2-sensitive [40]. If human P450 reductase is involved in microsomal Cr(VI) reduction, it must be much less O_2-sensitive than the rat enzyme.

Partial inhibition by *n*-octylamine [32,35] suggests a possible role for flavin-containing monooxygenase (FMO) (Table I)[46]. While *n*-octylamine acts as a type II inhibitor of certain P450s, the lack of inhibition by all other P450 inhibitors suggests that its effects are FMO-linked rather than P450-linked [75]. However, evidence using human FMO3 derived from an *E. coli* expression system indicate that FMO is not a significant mediator of Cr(VI) reduction (see below).

A relative insensitivity to O_2 is also a general property of the human microsomal Cr(VI)-reducing system, with room air (21% O_2) causing only a 26–37% inhibition relative to anaerobic conditions (Table I)[46]. This is in marked contrast to microsomal Cr(VI) reduction in rats which is markedly inhibited by 0.1 % O_2, and completely inhibited by 1% O_2 [38,39]. This implies that human microsomal Cr(VI) reduction would still proceed at significant rates even in tissues with high O_2 tensions. The limited O_2-sensitivity of the human system could imply the existence of more than one Cr(VI)-reducing enzyme, at least one of which is O_2-insensitive and at least one which is fully O_2-sensitive. Alternatively, there could be a single Cr(VI)-reducing system which is only partially inhibited by O_2. At this point, we cannot distinguish between the two possibilities.

Methods Used to Accomplish Aims #1 and #2:

Human Tissue. Human liver and lung tissues were provided by the Organ Transplant Unit at Froedtert Memorial Lutheran Hospital (Milwaukee, WI), with the approval of the Human Research Review Committee of the Medical College of Wisconsin. In all cases, the organs were removed from brain-dead organ transplant donors by the Organ Transplant Unit within approximately 30 min after death. The tissue was immediately iced, cut into small pieces, flash frozen in liquid nitrogen, and stored at –80°C as previously described [53]. Liver tissue from five humans (patients B, C, F, N, and O) and lung tissue from one (patient O) was used. A partial characterization of the P450 isozyme contents of the hepatic microsomes from four of these subjects has been published [9,73]. The pertinent patient data are as follows [73]: human B, male, age 50, no known drug history, heavy smoker; human C, male, age 22, ethanol detected; human F, female, age 50, alcoholic, diabetic, heavy smoker; human N, male, age 21, ethanol detected (0.087%), unknown smoking history; human O, male, age 49, unknown history.

Microsomal fractions were prepared from human tissue using a conventional differential centrifugation procedure as previously described [53].

Cr(VI) reduction assay. Experiments to assess Cr(VI) reductase activity were conducted as previously described [53], using an NADPH-regenerating system (3 mM $MgCl_2$, 1 mM NADP, 7 mM G6P, and 0.4 U G6P dehydrogenase ml^{-1}) at 37°C. Microsomes were pre-incubated for 5 min prior to the addition of Na_2CrO_4 to a final concentration of 19.6 μM. Net enzymatic rates were obtained by subtracting the slow rates (probably the result of a slow chemical reduction by NADPH [38]) that were observed in the presence of pre-boiled microsomes (which were previously shown to lack enzymatic activity [53]). The reduction of Cr(VI) was stopped by the addition of 125 μl 2 M Na_2CO_3 (per 2.5 ml reaction volume) [38]. The concentration of remaining Cr(VI) was measured colorimetrically by 1,5-diphenyl-carbazide (DPC) in acid solution (pH 2) [57] after removal of interfering reducing material by a charcoal/aluminum oxide mixture [56]. Cr(VI) concentrations were determined from a standard curve with Na_2CrO_4 as the standard by measuring absorbance at 540 nm against a blank in which Na_2CrO_4 was omitted. In some

experiments, the NADPH-dependence of the reduction of Cr(VI) was assessed by removing individual components of the NADPH-generating system.

Experiments to examine the kinetics of microsomal Cr(VI) reduction were performed in an analogous manner, except that different concentrations of Na_2CrO_4 were used and all reactions were stopped after 3 min.

Experiments under anaerobic conditions (4 to 5% H_2/balance N_2) were conducted in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI). All buffers, etc., were pre-incubated in the anaerobic chamber for at least one hour before use. Cr(VI) reduction rates under these conditions were found to be indistinguishable from those in which the vials were made anaerobic by flushing with O_2-free N_2. For those experiments conducted under aerobic conditions, open-top vials were incubated in a shaking waterbath (100 rpm) during all steps; for these vials, the reagents were not pre-incubated under anaerobic conditions.

Effects of Potential Inhibitors on Cr(VI) Reduction. The effect of CO on Cr(VI) reduction was examined as follows: microsomal preparations that had been rendered anaerobic were gassed with 100% CO on ice for 2 min and then pre-incubated for 5 min at 37°C prior to addition of Na_2CrO_4 in the Cr(VI) reduction assay. Controls were gassed under identical conditions with O_2-free N_2, which was generated by passing 100% high purity dry grade N_2 through an Oxi-Clear cartridge (Labclear, Oakland, CA).

For the other inhibitor experiments, the inhibitor was added at the same time as the NADPH-generating system and was therefore exposed to the microsomes during the 5 min pre-incubation period. The following stock solutions were made just before use: *n*-octylamine (0.3 M), aminopyrine (0.3 M), and piperonyl butoxide (6 mM) were prepared in DMSO; $TlCl_3$ (13.1 mM) and metyrapone (6.25 mM) were prepared in 10 mM HEPES, pH 7.5. Final inhibitor concentrations were as indicated in the results. For each inhibitor, Cr(VI) reduction rates were compared to those in the presence of the inhibitor solvent only.

Pretreatment of microsomes with 0.2 mM 2-bromo-4′-nitroacetophenone (BNAP), which inhibits microsomal NADPH:cytochrome *c* reductase, was done as described [21], and compared against controls in which BNAP was omitted.

Miscellaneous Procedures. Microsomal protein concentrations were determined by the Lowry method [33] modified as described to remove potential interference from detergents [13], with bovine serum albumin as the standard. Total cytochrome P450 content was determined from CO-reduced minus reduced spectra as described [49] using an Aminco DW-2000 spectrophotometer (SLM-Aminco Instruments, Urbana, IL); the difference in absorbances at 450 and 490 nm was used to calculate the molar concentration of P450, using an extinction coefficient of 91 mM^{-1} cm^{-1} [15]. NADPH:cytochrome P450 reductase activity was measured at 37°C as cytochrome *c* reduction [70]; the change in absorbance at 550 nm was followed over time in a 1-ml volume containing 0.1 μmol NADPH, 34 nmol cytochrome *c* "type III", 50 mM potassium phosphate (pH 7.7), 1 mM cyanide, and 0.1 to 0.5 mg microsomal protein (ε = 29.5 mM^{-1} cm^{-1} for reduced cytochrome *c*).

The rate of formation of NADPH in the presence of the NADPH-regenerating system (3 mM $MgCl_2$, 1 mM NADP, 7 mM G6P, and 0.4 U G6P dehydrogenase ml^{-1}) was measured at 37°C; the change in absorbance at 340 nm (ε = 6.1 mM^{-1} cm^{-1} for NADPH) was followed over time in a 2.5-ml reaction volume containing 150 mM KCl/25 mM HEPES, pH 7.35.

Aims #3 and #4, Accomplishments

Role of Iron in Human Microsomal Cr(VI) Reduction. While not an original aim or hypothesis of this project, a significant role for iron in human microsomal Cr(VI) reduction was discovered serendipitously [46]. The initial observation was that different salts and suppliers of glucose-6-phosphate (G6P), a component of the NADPH-generating system for the Cr(VI) reduction assay, each supported a different rate of Cr(VI) reduction by human hepatic microsomes. Extensive trial-and-error investigation revealed that there were significant differences in the iron content of the various G6Ps, and those with higher iron content were associated with higher rates

of microsomal Cr(VI) reduction (Fig. 1); while the G6P contributed a large part of the total iron in the Cr reduction assays, the rates of Cr(VI) reduction correlated most strongly with the total iron present and not just that contributed by G6P (Fig. 1) [46]. The addition of other forms of exogenous iron (e.g. $FeCl_3$, $FeSO_4$) to the microsomal Cr(VI) reduction experiments similarly enhanced the rates of Cr(VI) reduction (Figs. 2, 3). Additions of $FeCl_3$, which resulted in total iron amounts of 1.93 to 16.1 nmol Fe, caused marked increases in Cr(VI) reduction rates, while additional increases in total Fe (16.1 to 44.1 nmol) had little to no effect on further increasing Cr(VI) reduction rates (Fig. 2)[46]. The trend was similar for additions of $FeSO_4$; a change in total iron from 2.13 to 9.96 nmol markedly increased the rate of microsomal Cr(VI) reduction, and a further increase to 18.5 nmol Fe caused an incremental but less pronounced increase in Cr(VI) reduction rate (Fig. 3). Further additions of $FeSO_4$ to increase total Fe from 18.5 to 50.8 nmol did not result in further significant increases in Cr(VI) reduction (Fig. 3)[46]. The maximal rates of Cr(VI) reduction are approximately one-third greater for $FeSO_4$ addition (Fig. 3) relative to $FeCl_3$ addition (Fig. 2). Other studies have noted that certain rodent microsomal enzymes display differential rates of Fe reduction, depending on the nature of the Fe complex [42,44,68].

The Cr(VI) reduction rates seen with high levels of $FeCl_3$ (Fig. 2) were similar to those that were obtained using monopotassium-G6P in our initial experiments; the high iron content of experiments that included monopotassium-G6P (Fig. 1) are consistent with these higher Cr(VI) reduction rates. Since redox transformations of both iron and chromium can lead to oxidative damage [62], the ability of low concentrations of iron to markedly stimulate Cr(VI) reduction could have significant consequences. The levels of iron that markedly stimulated human microsomal Cr(VI) reduction rates (0.76–6.3 μM) are 3.1- to 26-fold lower than the initial Cr(VI) concentration in our assays. This level of iron is consistent with levels reported in other studies for which iron was shown to contribute to cell-damaging processes [10]. While it is generally accepted that the majority of intracellular iron is sequestered in ferritin, it is clear that there are intracellular pools of iron, distinct from ferritin, that are directly involved in mediating oxidative cell injury; these pools represent from 2–15% of total cellular iron, depending on various conditions [54].

Since the stimulatory Fe levels were far below the initial Cr(VI) concentration, it seems likely that Fe is serving a catalytic role. Because Fe(II) can directly reduce Cr(VI) [41,51], one possible scenario is that a small amount of Fe is repeatedly redox-cycled, resulting in the reduction of a much larger amount of Cr(VI). This has potentially significant toxicologic consequences, as the rate and extent of formation of reactive Cr intermediates could be greatly amplified by relatively small increases in intracellular iron. Since the oxidation of Fe(II) to Fe(III) by Cr(VI) represents a one-electron process, it seems likely that Cr(V) would be the resulting immediate product provided that Fe was limiting, as is the case for our human microsomal experiments. The formation of Cr(V) can lead directly to the generation of reactive oxygen species which are likely responsible for at least some of the damage associated with Cr(VI) exposure, e.g. DNA strand breaks, DNA-protein crosslinks, and lipid peroxidation [28,43,58,60]. Cr(V) can also catalyze the formation of lipid hydroperoxide radicals from previously existing lipid peroxides [59].

Since any one of several xenobiotics (e.g. paraquat, benzyl viologen, diquat, triphenyltetrazolium, nitrofurantoin, doxorubicin, daunomycin, diaziquone, anthraquinone 2-sulfonate) can mediate significant release of iron from ferritin [55,68], there could be significant toxicologic consequences for simultaneous exposure to Cr(VI) and one or more of these compounds. Similarly, both nitric oxide and superoxide can also mediate iron release from ferritin [55], so conditions which lead to increases in these free radical species could also potentially enhance Cr(VI) reduction rates and hence chromium-related cytotoxicity. In summary, the levels of iron for which we observe significant effects on Cr(VI) reduction are generally in line with levels at which iron is known to mediate other effects, and are within the range of what could be expected within a cell.

Since the initial characterizations of human microsomal Cr(VI) reduction (Table I) were done prior to the discovery of the effect of iron, Cr(VI) reduction parameters were reassessed using G6Ps with significantly different iron levels (Table II). These comparisons demonstrated that the concentration of iron markedly influenced the rate and V_{max} of Cr(VI) reduction (Table II; Figs. 2,

3). Other Cr(VI)-reducing parameters (e.g. K_m, inhibition by *n*-octylamine) were only influenced to a small extent by large differences in the concentration of iron (Table II).

Since Fe(II) can be readily oxidized by O_2 [4], additional experiments were done to determine the effects of O_2 on Cr(VI) reduction at various iron concentrations. The ratio of human microsomal Cr(VI) reduction rates under aerobic vs. anaerobic conditions remained fairly constant, regardless of iron concentration (Fig. 4)[46]. Even though iron varied from 1.93 to 16.1 nmol, aerobic Cr(VI) reduction rates for human O were 63–71% of anaerobic rates (Fig. 4). For human N, a similar ratio range was observed. This is consistent with our findings that human microsomal Cr(VI) reduction still proceeds at significant rates under room air (Table I). Theoretically, one might expect that O_2 would markedly inhibit both Cr(VI) and iron reduction, as is the case in rats [38,39,68]. While O_2 could theoretically inhibit any role for iron by rapidly oxidizing Fe(II) to Fe(III), it is known that, below pH 10, the rate of Fe(II) oxidation by Cr(VI) is greater than the rate of Fe(II) oxidation by O_2 [51]. This is consistent with our findings in humans that any inhibition of Cr(VI) reduction by O_2 is independent of the iron level (Fig. 4). Hence, in humans, O_2 should have same effect regardless of iron level, i.e. iron can significantly stimulate Cr(VI) reduction rates under both anoxic and fully oxic conditions.

The absolute rates of Cr(VI) and Fe(III) reduction were compared for hepatic microsomes from three humans, and in all cases the rate of Fe(III) reduction was marginally greater than the rate of Cr(VI) reduction (Table III)[46]. Theoretically, the rate of reduction of Fe(III) is therefore sufficient to support the rate of Cr(VI) reduction (Fig. 5). At this point, however, it is unclear whether human microsomal Cr(VI) reduction is totally linked to iron, or whether some portion of the activity is not dependent on iron. It is impossible to completely exclude iron from these experiments (e.g. microsomes contain iron in various forms, including that contained in heme groups, iron-sulfur centers, etc.). However, if one extrapolates the lines in Figures 2–4 back to zero iron, all of the lines imply some Cr(VI)-reducing activity in the absence of iron. This extrapolated "iron-independent" activity comprises 13–30% of the maximal Cr(VI)-reducing activity under high iron for those experiments in which $FeCl_3$ served as the source of exogenous iron, and 8–15% of the maximal Cr(VI)-reducing activity for those experiments in which $FeSO_4$ served as the source of exogenous iron [46].

It is known that certain iron chelates can increase the rate of NADPH-linked Cr(VI) reduction by rat microsomes, and it has been suggested that the mechanism is based on the redox cycling of iron [41]. This is consistent with our proposed mechanism by which Fe may be stimulating the Cr(VI) reduction rates by human microsomes. However, the extrapolation of these rodent studies to humans is not necessarily direct, as there are several discrepancies or differences from our human studies. First, the rat studies utilized Fe(III)-EDTA, whereas $FeCl_3$, $FeSO_4$, or an iron contaminant in G6P served as the source of iron for the human studies reported here. Human microsomes could also efficiently reduce Fe(III) citrate (Table III). Second, the rat studies required very high levels of iron chelates (50 – 150 μM), which were 1.7- to 5-fold higher than the level of Cr(VI) [41]. In contrast, in our human studies, Cr(VI) reduction was stimulated by Fe in the range of 0.76–6.3 μM which is 3.1- to 26-fold lower than initial Cr(VI) levels. Hence, human Cr(VI) reduction rates are stimulated by iron at levels that are 8- to 197-fold lower than those used in the rat studies. While the exact mechanism of Cr(VI) and/or Fe(III) reduction in human microsomes is not yet clear, the effects of iron on the rate of Cr(VI) reduction occur at much lower iron concentrations in humans vs. rats. Iron is thus much more likely to play a significant role in Cr(VI) reduction in humans because these lower iron concentrations are likely to be more readily achieved.

Since the lungs are also a potential target organ for chromium toxicity, the effect of iron on the rate of Cr(VI) reduction by lung microsomes from patient O was determined. Lung microsomal Cr(VI) reduction rates of 0.81 ± 0.03 and 0.16 ± 0.02 nmol/min/mg protein were observed for experiments in which the total iron content was 4.86 and 1.54 nmol, respectively [46]. While this relative effect of iron is similar to what was observed using liver microsomes from the same patient, the Cr(VI) reduction rates catalyzed by lung microsomes were lower than those obtained using hepatic microsomes. This is consistent with the lower V_{max} for Cr(VI) reduction observed with lung microsomes (3.6 ± 0.7 nmol/min/mg), which is approximately one-third of the hepatic V_{max} in this patient.

This pronounced iron effect necessitated an increased complexity for subsequent Cr(VI) reduction experiments, such that we had to also examine iron reduction as an indirect means by which Cr(VI) could be reduced. Details of how this was applied to testing the activity of purified proteins are included below. The pronounced effect of iron also largely negates the reasoning for pursuing aim #3 at this time. The original idea behind aim #3 was that Cr(VI)-reducing enzymes would likely be subject to direct attack by the reactive Cr intermediates, Cr(V) and/or Cr(IV), because of their intimate proximity with these intermediates as they are formed. However, since much of the Cr(VI) reduction seems to be mediated through iron reduction, the reactive Cr intermediates do not have to be formed in close association with the iron-reducing enzymes. The likelihood of preferential free radical attack on the responsible enzymes is therefore greatly diminished, and pursuit of this aim would be better left until we have reconstituted purified enzyme mixes in hand.

Role of FMO in Cr(VI) Reduction. Inhibitor data (above) implicated flavin-containing monooxygenase (FMO) as a significant contributor to microsomal Cr(VI) reduction. Since the major human FMO isoform, FMO3, has not been successfully purified from human liver, a variety of approaches were utilized to develop the tools (e.g. antibodies, purified proteins, etc.) necessary for assessing the ability of FMO to mediate Cr(VI) reduction. The first approach was to generate an antipeptide antibody specific for human FMO3 [48]; while it reacted well in Western blots and is therefore useful for the immunoquantification/detection of this FMO, it reacted poorly with the native conformation of FMO3 and was therefore not useful for the inhibition or immunopurification of FMO3 [48].

As an alternate approach, a bacterial expression system was developed. The cDNA for FMO3, generated by PCR using specific primers and a human liver cDNA library template, was cloned into the expression vector pCALc (Stratagene) which contains an in-frame calmodulin-binding domain (CBD) at the C-terminus. After successfully optimizing conditions for expression of the FMO3 fusion from this clone, sufficient FMO3-CBD fusion was purified for use as an antigen, and to test its activity. While the antibody against this fusion is specific for FMO in Western blots, it does not inhibit the FMO activity of human microsomes. In addition, the FMO3-CBD fusion did not display significant FMO activity (oxidation of methyl *p*-tolyl sulfide), so it could not be used directly to assess Cr(VI)- or Fe(III)-reducing activity.

An alternate bacterial expression approach was therefore developed, based on cloning the human FMO3 into pMALc (New England BioLabs), which contains an in-frame maltose-binding protein (MBP) at its N-terminus [5]. After successfully optimizing conditions for expression of the FMO3 fusion from this clone, sufficient FMO3-MBP fusion was purified for use as an antigen, and to test its activity. This fusion protein displayed prominent FMO activity (oxidation of methyl *p*-tolyl sulfide), but could not reduce Cr(VI) or any of the Fe(III) complexes that were examined. This suggests that FMO3 is not a significant player in human microsomal Cr(VI) reduction. However, the possibility remains that a post-translational modification of FMO3 in human tissue is required for metal reduction. We have plans to pursue this possibility using the antibody generated against this fusion. While this antibody does not inhibit the FMO activity of human microsomes, it should be useful for immunoaffinity chromatography [72] as a means for specific removal of FMO from solubilized microsomes. It is also possible that other FMO isoforms (e.g. FMO2 in lung) could play a role in Cr(VI) or Fe(III) reduction.

Role of P450 Reductase and Cytochrome b_5 in Cr(VI) Reduction. Inhibitor data (above) suggest a prominent role for P450 reductase in human microsomal Cr(VI) reduction). To further assess the role of human P450 reductase in Cr(VI) reduction, we used steapsin which is known to remove the 72-kDa catalytic domain of P450 reductase from microsomes [21] (Fig. 6). Steapsin does not impair the catalytic activity of purified FMO or deplete rabbit liver microsomes of FMO [21], and protease treatment does not remove cytochrome b_5 reductase from rat microsomes [50]. Treatment of hepatic microsomes with pre-boiled (inactive) steapsin left essentially all of the P450 reductase and Cr(VI) reductase activities associated with the pellet as expected (Table IV). In contrast, active steapsin removed 94–96% of the P450 reductase activity and 66–68% of the

Cr(VI) reductase activity from the microsomal pellet (Table IV). The P450 reductase activity was fully accounted for in the resulting supernatant fraction (as NADPH:cytochrome *c* reductase activity), but no Cr(VI) reductase activity was present in this supernatant. This suggested that either the released P450 reductase was no longer capable of reducing Cr(VI), or that it was part of a two component system and the other component remained with the microsomal pellet. To test for this latter possibility, when we re-combined the post-steapsin supernatant and pellet fractions, the Cr(VI) reductase activity was restored (Table IV), and to a level even greater than expected. Steapsin itself has no Cr(VI) reductase activity to explain this, nor does it contain detectable iron which could stimulate Cr(VI) reduction (see above). It seems most likely that the greater than expected activity was because the P450 reductase is no longer tethered to the microsomes via its N-terminal anchor and was therefore able to more efficiently associate with other microsomal components. Other reports have noted that the lipase- or protease-cleaved P450 reductase can behave very differently from that anchored to membranes [14], and that the NADPH:cytochrome *c* reductase activity of P450 reductase does not always correlate with its ability to interact with other potential electron acceptors [36,52].

Thus, it seems that the human P450 reductase by itself cannot mediate Cr(VI) reduction, but acts in concert with other as yet unidentified microsomal component(s). One such possible component is cytochrome b_5, which can accept electrons from P450 reductase [14]. This is supported by the observation that when we added purified recombinant cytochrome b_5 to human N microsomes, the rate of Cr(VI) reduction more than doubled (Table V)[46]. Additional studies demonstrated that the response to cytochrome b_5 was linear, i.e. the more cytochrome b_5 added, the higher the Cr(VI) reduction rate (Fig. 7).

Several approaches were utilized to develop the tools necessary to explore the role of P450 reductase in chromium and iron reduction. An antipeptide antibody was generated against the human P450 reductase, but its specificity was not absolute for this protein. A bacterial expression system approach, analogous to that used for FMO (above), was utilized for human P450 reductase. After optimizing conditions for its expression, sufficient material was purified, using affinity chromatography, for use as an antigen and in activity studies.

Purified P450 reductase is able to directly reduce iron and chromium, although the observed rates of reduction are quite low, especially when compared to its ability to reduce cytochrome *c* (Table VI). When normalized per unit of cytochrome *c* reductase activity, purified P450 reductase is a relatively poor reducer of iron or chromium when compared to human microsomes, i.e. human microsomes can reduce Cr(VI) and Fe(III) 71-fold and 55-fold faster, respectively, than purified P450 reductase (Table VI). This agrees with our previous results that suggest that P450 reductase, by itself, is a poor reducer of iron and chromium, and that it likely acts in concert with other microsomal components, e.g. cytochrome b_5. As was the case with microsomes (Tables III, VI), the Fe(III) reductase rate of purified P450 reductase was greater than its Cr(VI) reductase activity, suggesting that the rate of Fe(III) reduction is sufficient to support the rate of Cr(VI) reduction.

Polyclonal antisera raised against purified P450 reductase inhibited its ability to reduce cytochrome *c* by approximately 50%. Similarly, this antibody caused a ~50% inhibition of NADPH:cytochrome *c* reductase activity in human microsomes, and it inhibited their ability to reduce Fe(III) by 23–32%. This antibody did not inhibit Cr(VI) reduction by microsomes, but since most microsomal activity is dependent on iron, a partial inhibition of iron reduction would not likely be sufficient to cause a significant inhibition of Cr(VI) reduction (Table III).

Purified human cytochrome b_5 is now available commercially (PanVera), and can be used to examine the potential cooperation of cytochrome b_5 and P450 reductase in the reduction of chromium and iron. While these proteins do not interact efficiently in solution, we will incorporate them into liposomes for these studies.

If cytochrome b_5 is involved, then it could also potentially mediate Cr(VI) and/or Fe(III) reduction in cooperation with cytochrome b_5 reductase in an NADH-dependent process. To explore this possibility, a bacterial expression system approach, analogous to that used for FMO and P450 reductase (above), was utilized for human cytochrome b_5 reductase. After optimizing conditions for its expression, sufficient material was purified, using affinity chromatography, for use as an antigen and in activity studies. On its own, cytochrome b_5 reductase cannot mediate

Cr(VI) or Fe(III) reduction at significant rates. This purified protein will be used in future studies to explore the potential cooperative interaction between cytochrome b_5 and b_5 reductase in the reduction of Cr(VI) and Fe(III). The antibody to b_5 reductase is able to partially inhibit b_5 reductase activity, so it should be useful in these studies as well.

As noted above, we observed that Cr(VI) reduction rates catalyzed by lung microsomes were approximately 25-33% of those for liver. This is consistent with the fact that the levels of P450 reductase and cytochrome b_5 in human lung are approximately 20–25% of those in human liver [37], and with our data that suggest that P450 reductase and cytochrome b_5 are involved in human microsomal Cr(VI) reduction. Since the P450 reductase and cytochrome b_5 expressed in various tissues are apparently identical (i.e. there are not different isoforms in different tissues [20,31]), it seems likely that these enzymes could also contribute to Cr(VI) reduction in non-hepatic tissues, so long as they are present at adequate levels. When taking into account that, in the lung, P450 reductase is primarily localized in bronchial and bronchiolar epithelial cells [20], it seems likely that the concentration of P450 reductase in these bronchial epithelial cells is as great or greater than that in hepatic cells and could therefore make a significant contribution to Cr(VI) reduction. While these epithelial cells comprise a minority of the cells in the lung, they are major sites of exposure from inhaled Cr(VI) and of pulmonary tumors including those related to Cr exposure. However, the contribution of microsomal enzymes, relative to other enzymatic and non-enzymatic cellular components, to total cellular Cr(VI) reduction remains to be determined.

Preliminary ESR Studies During Human Microsomal Cr(VI) Reduction. In preliminary experiments, ESR (electron spin resonance) spectra collected at 77 K clearly showed the production of the reactive intermediate Cr(V) (distinct signal with g value of 1.98) during the ascorbate-mediated reduction of Cr(VI) (Fig. 8, top). A shallow broad signal spanning the distinct Cr(V) signal was also seen during the ascorbate-mediated reduction of Cr(V) (Fig. 8, top); this broad signal (peak-to-peak width of approximately 750 G) could represent either Cr(IV) [34] or Cr(III). Cr(V) was also clearly evident during the reduction of Cr(VI) by human hepatic microsomes (Fig. 8, middle). Upon expansion of the range from 4000 G to 100 G, asymmetry in the g = 1.98 line was observed (data not shown). A much smaller Cr(V) signal was seen when pre-boiled microsomes were used (Fig. 8, bottom); this was probably due to the exposure to the NADPH-generating system for 60 min, as NADPH is a known reductant of Cr(VI) [19,23,60] although at a much slower rate than that catalyzed by microsomal enzymes [53]. NADPH-generating systems have been previously shown to generate small amounts of Cr(V) readily detectable by ESR [1]. The relative Cr(V) signal intensities indicate that the signal with pre-boiled microsomes was only 43% of that with active microsomes, consistent with an active role for microsomes in generating Cr(V). Even though these were conducted in the absence of O_2, some of the Cr may be redox-cycled, and so the relative signal intensities may not represent absolute amounts of Cr(V) produced, but rather relative "steady-state" levels under the specific experimental conditions. We have not yet examined other experimental conditions that might affect ESR signals, including alternate incubation times, altered concentrations of various components, NADPH-free controls, etc.

The sum total of our findings has led to the current working hypothesis of human microsomal Cr(VI) reduction as presented in Fig. 9. Briefly, a role for cytochromes P450 has been eliminated, and while all possibilities have not been exhausted, it would seem that FMO3 does not have a significant role in Cr(VI) or Fe(III) reduction. P450 reductase is required for approximately two-thirds of total Cr(VI) reducing activity, but on its own is a poor reducer of Cr(VI) and Fe(III). P450 reductase likely acts in concert with other microsomal components, such as cytochrome b_5 or other as yet unidentified electron carriers. If cytochrome b_5 is involved, it can also likely cooperate with cytochrome b_5 reductase to mediate Cr(VI) and/or Fe(III) reduction in a NADH-dependent manner. While all possibilities have not been exhausted, it seems that cytochrome b_5 reductase, by itself, does not appear to have a significant role in Cr(VI) or Fe(III) reduction.

Methods Used to Accomplish Aims #3 and #4:

Determination of the Iron Content of Solutions. To determine the iron content of G6Ps and other solutions used in the chromium reduction assay, solutions were mixed with freshly prepared sodium hydrosulfite (final concentration, 10 mM) to reduce all iron to Fe(II). Different aliquots (50–500 μl) of these solutions were then processed through the ferrozine assay [47,63] to determine the concentration of Fe(II). Calculations were made to account for volume differences to determine the amount of Fe contributed by each solution. No iron was detected in the 10 mM sodium hydrosulfite solution.

Reduction of Ferric Compounds by Microsomes. Ferric reductase activity was assayed under anaerobic conditions by monitoring the production of Fe(II) over time as adapted from Myers and Myers [45]. All solutions were pre-incubated in the anaerobic chamber for at least one hour before use. The assays were performed at 37°C in temperature-controlled stirred anaerobic cuvettes using an Aminco anaerobic cell accessory (SLM Instruments, Urbana, IL); the assay mix (3.0 ml total volume) consisted of: 28 mM potassium phosphate buffer (pH 7.5), 0.4 mM ferrozine, 0.12 mM ferric citrate, and 0.18–0.26 mg of microsomal protein. The anaerobic cuvettes were set up in an anaerobic chamber in a 37°C heating block, and sealed before removal from the chamber to maintain anaerobic conditions. The assay was started by the addition of 60 μl 50 mM anaerobic NADPH to the sample cuvette (using the sealed plunger device of the anaerobic cell accessory); the reference cuvette received 60 μl anaerobic water at the same time. Activity was followed by an increase in absorbance over 3 min at 562 nm. Rates of Fe(III) reduction in the absence of microsomes, which were minimal, were subtracted from microsomal rates to obtain the net values due to microsomal activity. Using the extinction coefficient for the ferrozine-Fe(II) complex of 28 mM^{-1} cm^{-1} [8], changes in absorbance were converted to nmol of ferrozine-Fe(II) complex formed (equivalent to nmol Fe(III) reduced) per min at 37°C. In some experiments, other iron compounds were substituted for ferric citrate, including ferric-ATP, ferric-ADP, ferric EDTA, ferric ammonium sulfate, ferric chloride, ferric DETPA, ferric EDDA, and ferric acetylacetonate.

Determination of Enzyme Activities. FMO3 activity was assessed as oxidation of methyl *p*-tolyl sulfide using a method adapted from Brunelle et al. [5]. Specifically, the reaction was conducted in 50 mM potassium phosphate (pH 8.4) containing an NADPH-generating system (0.5 mM NADP, 2.0 mM G6P, 2 U G6P dehydrogenase ml^{-1}), 0.8 mM DETAPAC, 1 μM FAD. The FMO3 source was added and incubated with slow shaking for 2 min at 37°C. Methyl *p*-tolyl sulfide was added to a final concentration of 0.5 mM, and incubation was for 40 min at 37°C. Final total reaction volume was 0.25 ml. The reaction was stopped by adding 0.70 ml ice-cold acetonitrile, after which the tube was briefly vortexed and immediately iced. 20 mg NaCl was added to each tube, after which the tube was extensively vortexed. Following a 10 min centrifugation at 2000 x g at 4°C to separate the phases, the organic layer was analyzed by isocratic HPLC (mobile phase: 50% acetonitrile in water, 1.0 ml/min; Alltech C8 RSIL column, 250 x 4.6 mm; UV detection at 220 nm). The substrate (methyl *p*-tolyl sulfide) and product (methyl *p*-tolyl sulfoxide) were quantified by comparison of peak areas relative to those of standards of these two compounds.

NADH:cytochrome b_5 reductase activity was determined as NADH-dependent ferricyanide reduction as described [69,74].

NADPH:cytochrome P450 reductase activity was measured at 37°C as cytochrome *c* reduction [70]; the change in absorbance at 550 nm was followed over time in a 1-ml volume containing 0.1 μmol NADPH, 34 nmol cytochrome *c* "type III", 50 mM potassium phosphate (pH 7.7), 1 mM cyanide, and 0.1 to 0.5 mg microsomal protein (ε = 29.5 mM^{-1} cm^{-1} for reduced cytochrome *c*).

Cr(VI) reduction was determined as described above except that for experiments involving b_5 reductase, NADH was substituted for the NADPH-generating system.

NEW DISCOVERIES, CUMULATIVE:

1. There is little difference in chromium-reducing rates or properties when comparing hepatic microsomes from five individuals.
2. The kinetic properties of chromium reduction are consistent with a potential significant role for *in vivo* Cr(VI) reduction, using Cr(VI) concentrations well below those anticipated for occupational exposure (apparent K_m for chromate of 1.04–1.68 μM).
3. Relative to anaerobic conditions, Cr(VI) reduction was inhibited only 26–37% by room air, suggesting that human microsomal Cr(VI) reduction could still proceed at significant rates even in tissues with high O_2 tensions.
4. Studies with lung microsomes from one human exhibited V_{max} and K_m values that were two-thirds lower and 2.8-fold greater, respectively, than those of hepatic microsomes from the same individual; other Cr(VI)-reducing parameters were similar for lung and liver.
5. Human microsomal Cr(VI) reduction is not catalyzed by cytochromes P450.
6. A significant role for flavin-containing monooxygenase isoform 3 (FMO3) in Cr(VI) or Fe(III) reduction is not apparent.
7. Human cytochrome P450 reductase plays a significant role in microsomal Cr(VI) reduction, but by itself is a relatively poor reducer of Cr(VI) and Fe(III). It apparently acts in concert with other microsomal components, e.g. cytochrome b_5, to mediate significant rates of Cr(VI) reduction.
8. Cytochrome b_5 reductase does not appear to directly mediate Cr(VI) or Fe(III) reduction at significant rates, but could play a significant role by acting in concert with cytochrome b_5.
9. A strong link between the chromium and iron reductase activities of human microsomes was identified. Specific aspects of this relationship are as follows:
   a. The levels of iron that markedly stimulated human microsomal Cr(VI) reduction rates were 3.1- to 26-fold lower than the initial Cr(VI) concentration, suggesting that iron is serving a catalytic role, i.e. a small amount of iron is repeatedly redox-cycled, resulting in the reduction of a much larger amount of Cr(VI). This has potentially significant toxicologic consequences, as the rate and extent of formation of reactive Cr intermediates could be greatly amplified by relatively small increases in intracellular iron..
   b. The concentration of iron markedly influenced the rate and V_{max} of Cr(VI) reduction, whereas other Cr(VI)-reducing parameters were only influenced to a small extent by large differences in the concentration of iron.
   c. Iron can significantly stimulate Cr(VI) reduction rates under both anoxic and fully oxic conditions. Hence, the effect of iron should not vary based on the relative oxygenation of tissues.
   d. The rate of reduction of Fe(III) is theoretically sufficient to support the rate of Cr(VI) reduction. However, between 8 and 30% of total microsomal Cr(VI)-reducing activity may be independent of iron.
   e. The relative effect of iron on Cr(VI) reduction rates is similar for lung and liver microsomes.
   f. Since several other xenobiotics (e.g. paraquat, benzyl viologen, diquat, triphenyltetrazolium, nitrofurantoin, doxorubicin, daunomycin, diaziquone, anthraquinone 2-sulfonate) can mediate significant release of iron from ferritin, there could be significant toxicologic consequences for simultaneous exposure to Cr(VI) and one or more of these compounds.
10. Human hepatic microsomes generate the reactive intermediate Cr(V) during the reduction of Cr(VI). Since Cr(V) is known to lead directly to the generation of reactive oxygen species and the formation of lipid hydroperoxide radicals from previously existing lipid peroxides, this highlights the potential toxicologic consequences of microsomal Cr(VI) reduction.
11. Various antibodies were generated for use as tools in these studies:
    a. An antipeptide antibody specific for human FMO3 was generated which reacted well in Western blots and is therefore useful for the immunoquantification/detection of FMO3.

However, it reacted poorly with the native conformation of FMO3 and will therefore not be useful for the inhibition or immunopurification of FMO3.

b. An antibody was generated against a recombinant FMO3-MBP (maltose-binding protein) fusion. While it does not inhibit FMO activity, it should be useful for immunoaffinity purification of native human FMO3.
c. A polyclonal antibody was raised against purified recombinant P450 reductase. This antibody can partially inhibit the ability of P450 reductase to reduce cytochrome *c* and iron(III). It should also prove useful for immunoaffinity purification of native human P450 reductase.
d. A polyclonal antibody was raised against a recombinant cytochrome b_5 reductase-CBD (calmodulin-binding domain) fusion. The antibody is able to partially inhibit the ability of b_5 reductase to reduce ferricyanide, and should therefore be useful in activity studies, as well as for immunoaffinity purification of native human cytochrome b_5 reductase..

12. The sum total of findings support the original contention that the rodent model is not appropriate to understand the generation of reactive chromium intermediates catalyzed by human microsomal enzymes. Continuation with these human studies is therefore necessary to understand chromium(VI)-mediated toxicity and carcinogenicity in man.

LISTING OF COMMUNICATIONS AND INTERACTIONS:

Personnel Involved (1 APR 95 to 31 MAR 98):

Charles R. Myers, Ph.D., Principal Investigator. 1 APR 95 to 31 MAR 98. 30–50% effort.
Judith M. Myers, M.S., Research Scientist. 1 AUG 95 to 31 MAR 98. 50–100% effort.
Björn Porgilsson, B.S., Graduate Student. 3 JAN 96 to 30 JUN 96; 19 AUG 96 to 31 MAR 98. 20–100% effort (stipend provided by MCW Graduate School or Dept. of Pharmacology & Toxicology).
Paul Jannetto, B.S., Graduate Student. 1 JUL 97 to 31 MAR 98. 50–100% effort (stipend provided by AASERT/AFOSR grant linked to this grant and by MCW Graduate School).
Christina Spofford, B.S., Graduate Student. 1 JUN 95 to 10 AUG 95. 100% effort (stipend provided by MCW MSTP Program).
Linnea Dahl, B.S., Laboratory Technologist. 24 APR 95 to 25 JUL 95. 100% effort.

Publications (1 APR 95 to 31 MAR 98):

C.R. Myers. 1997. Subcellular sites of xenobiotic-induced free-radical generation. **In** *Free Radical Toxicology* (K.B. Wallace, ed.), pp. 25–44. Taylor & Francis, Washington, DC.
C.R. Myers, B. Porgilsson, and J.M. Myers. 1997. Antibodies to a synthetic peptide that react with flavin-containing monooxygenase (HLFMO3) in human hepatic microsomes. *J. Pharmacol. Toxicol. Meth.* 37: 61–66.
C.R. Myers and J.M. Myers. 1998. Iron stimulates the rate of reduction of hexavalent chromium by human microsomes. *Carcinogenesis* In press.

Meetings and Seminars (1 APR 95 to 31 MAR 98):

1. AFOSR Predictive Toxicology Program Review
   31 MAY to 1 JUN 1995, Dayton, OH
   Presented a seminar on this project.

2. AFOSR Predictive Toxicology Program Review
   11-13 DEC 1996, Dayton, OH
   Presented a seminar on this project.

Established collaborative ties for future research with other AFOSR-funded investigators, including Dr. Sidney Stohs, Drs. Frank Siegel and Steven Kornguth, and Dr. Gerald A. LeBlanc.

3. Seminar:
   15 MAY 1997, Dept. of Pharmacology & Toxicology, Medical College of Wisconsin
   Title: "Reductive Metal Metabolism: Emerging View of a Multi-Component Electron Transport Chain"
   The seminar was attended by members of various departments including Pharmacology/Toxicology, Physiology, Cell Biology, Anesthesiology, and others. The seminar resulted in a request by Dr. Marilyn Merker (Anesthesiology) that I collaborate with her on a project involving reductase enzymes exposed on the cell surface of lung endothelial cells (see below).

4. Seminar:
   20 AUG 1997, Dept. of Pathology, Medical College of Wisconsin
   Title: "Chromium Toxicity: Initial Insights into Reductive Activation by Human Enzymes"
   The seminar was attended by members of various departments including Pathology, Preventive Medicine, and others. The seminar resulted in discussions with Dr. William Greaves (Preventive Medicine) on areas for potential future collaboration regarding toxicity associated with occupational chromium exposure.

Interactions (1 APR 95 to 31 MAR 98):

1. A collaboration was established with William Antholine, Ph.D., Associate Professor of Biophysics, and member of the National Biomedical ESR Center at the Medical College of Wisconsin, who is an expert in obtaining and interpreting ESR spectra from a variety of compounds, including various metals. We conducted preliminary experiments which resulted in the discovery that human hepatic microsomes generate Cr(V), a reactive intermediate, during the enzymatic reduction of Cr(VI). As a result of these findings, Dr. Antholine was added as co-Principal Investigator on the renewal of this project which has been subsequently funded by AFOSR (grant no. F49620-98-1-0079). His addition will facilitate our ability to establish a direct link between the activity of particular Cr-reducing enzymes and the types of reactive intermediates formed. Furthermore, this aligns this project with one of the most highly respected ESR centers in the country.

2. I invited AFOSR-funded researcher Dr. Steven Kornguth (University of Wisconsin) to present a seminar in our department. This seminar entitled "Glutathione S-Transferases as Biomarkers for Toxicant Exposure" was on March 12, 1998. It further provided the opportunity for us to discuss each others' recent findings and explore potential areas of common interest, including potential biomarkers for chromium exposure.

Consultative and Advisory Functions (1 APR 95 to 31 MAR 98):

My research efforts and results on this project directly led to the following opportunities to serve as an advisor, consultant, or collaborator on several other projects:

1. My development and use of antipeptide antibodies and their use in immunoblotting provided me with an opportunity to serve as a collaborator with Dr. N.C. Bols and co-workers (University of Waterloo, Ontario, Canada). This collaboration has resulted in two publications on the induction of cytochrome P4501A1 by PCBs and dioxin:
   J. H. Clemons, L. E. J. Lee, C. R. Myers, D. G. Dixon, and N. C. Bols. 1996. Cytochrome P4501A1 induction by polychlorinated biphenyls (PCBs) in liver cell lines

from rat and trout and the derivation of toxic equivalency factors (TEFs). *Can. J. Fish Aquat. Sci.* 53:1177–1185.

J. H. Clemons, C. R. Myers, L. E. J. Lee, D. G. Dixon, and N. C. Bols. Induction of cytochrome P4501A1 by binary mixtures of polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in liver cell lines from rat and trout. Submitted to *Aquatic Toxicology*.

2. My expertise, equipment, and studies on the potential role of cytochrome P450s in human chromium reduction for the AFOSR-funded studies provided me with an opportunity to serve as a collaborator with Dr. William Campbell (Medical College of Wisconsin) to elucidate the binding of nitric oxide to mitochondrial P450s, and the subsequent inhibition of aldosterone synthesis. This collaboration has resulted in one publication:

   C.J. Hanke, J.G. Drewett, C.R. Myers, and W.B. Campbell. Nitric oxide inhibits aldosterone synthesis by a guanylyl cyclase-independent effect. Revision submitted to *Endocrinology*.

3. My expertise, equipment, and use of molecular biology techniques for the AFOSR-funded studies on human chromium reduction provided me with an opportunity to serve as a collaborator with Drs. Pin-Lan Li and William Campbell (Medical College of Wisconsin) to clone and sequence the cDNA for ADP-ribosyltransferase in bovine coronary artery smooth muscle. This collaboration has resulted in one publication in preparation:

   P.-L. Li, C.-L. Chen, R. Bortel, C.R. Myers, A.-P. Zou, and W.B. Campbell. Endogenous mono-ADP-ribosylation in bovine coronary arterial smooth muscle. In preparation.

4. My expertise and study of chromium-reducing enzymes in the AFOSR-funded studies resulted in a request that I serve as a co-investigator with Dr. Marilyn Merker (Dept. of Anesthesiology, Medical College of Wisconsin) to identify and characterize cell surface-exposed reductases on lung endothelial cells. This has resulted in submission of a grant proposal to NIH.

Technology Transfers (1 APR 95 to 31 MAR 98):

The following technology transfers are directly related to research tools derived from this project:

1. Customer: Dr. Dan Schlenk
   Department of Pharmacology
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   Result: As one of the tools needed for our AFOSR project, we developed an antipeptide antibody that specifically recognizes the major human hepatic flavin-containing monooxygenase (FMO3). We described the development of this antibody in a publication (J. Pharmacol. Toxicol. Meth. 37:61–66; 1997). On 18 JUL 97, some of this antibody was transferred to Dr. Schlenk.

   Application: Dr. Schlenk is using the antibody as a biomonitoring tool to detect FMO induction in aquatic species. This antibody could become a significant tool to utilize FMO induction as a biomonitor to detect various classes of bioavailable organic pollutants in aquatic environments.

2. Customer: Dr. Sonia de Morais
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Result: As one of the tools needed for our AFOSR project, we developed an antipeptide antibody that specifically recognizes the major human hepatic flavin-containing monooxygenase (FMO3). We described the development of this antibody in a publication (J. Pharmacol. Toxicol. Meth. 37:61–66; 1997). On 15 SEPT 97, some of this antibody was transferred to Dr. de Morais.

Application: Dr. de Morais is using the antibody in Western blots to study the induction of FMO3 in cultured human hepatocytes treated with a variety of compounds. FMO3 is a major contributor to the metabolism/detoxification of hundreds of foreign compounds; her use of this antibody will greatly expand our understanding of FMO induction and of the potential role of human FMO3 in the metabolism of various xenobiotics.

Note: The anti-FMO3 antibody is also available to any Air Force personnel who wish to use it.

Inventions (1 APR 95 to 31 MAR 98):

No inventions.

Honors/Awards (1 APR 95 to 31 MAR 98):

None.

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TABLES AND FIGURES

Table I. Cr(VI)-reducing parameters of hepatic microsomes from five humans

Parameter	Observation[a]
Microsomal	YES
NADPH-dependent	YES
Inhibition by P450 inhibitors:	
carbon monoxide (100%)	none
piperonyl butoxide (0.12 mM)	none
metyrapone (50 μM)	none
aminopyrine (5.9 mM)	none
K_m, μM Cr(VI)	1.04–1.68
V_{max} (nmol/min/mg)	10.4–10.7[b]
Inhibition by O_2 (21%)	26–37%
Inhibition by $TlCl_3$ (0.26 mM)	96–100%
Inhibition by *n*-octylamine (5.9 mM)	19–28%
Inhibition by BNAP[c] (0.2 mM)	80–85%

[a] For numerical data, values represent the full range of results, from lowest to highest. For P450 inhibitor data, "none" indicates no measurable inhibition for any of the human microsomes.

[b] Values for human N were not included. In a previous experiment, the V_{max} for human N was 5.03 nmol/min/mg. This was obtained using a different lot of G6P for which the iron content was not determined (see below). In a recent repeat using iron levels comparable to those of the experiments with the other individuals, we obtained a V_{max} of 10.8 ± 1.05 nmol/min/mg protein for human N.

[c] BNAP, 2-bromo-4'-nitroacetophenone.

Table II. A comparison of key Cr(VI)-reducing parameters of human O hepatic microsomes in experiments utilizing G6Ps with different levels of iron

Parameter	G6P Source	
	K[a]	K_2[b]
NADPH dependence	Yes	Yes
K_m, μM Cr(VI)	1.68 ± 0.23[c]	2.33 ± 0.23[d]
V_{max} (nmol/min/mg)	10.40 ± 0.55[c]	1.86 ± 0.39[d]
% Inhibition by *n*-octylamine	28.0 ± 4.1[d]	40.3 ± 2.5[d]

a Monopotassium G6P; 7.8 nmol total Fe present in assays.
b Dipotassium G6P; 2.3 nmol total Fe present in assays.
c Results are mean ± S.D., $n = 3$.
d Results are mean ± S.D., $n = 2$.

Table III. Comparison of Cr(VI) and Fe(III) reductase activities of human hepatic microsomes

Microsomal Source	Specific Fe(III) Reductase (nmol/min/mg)[a]	Specific Cr(VI) Reductase (nmol/min/mg)[b]
Human C	6.96	5.00
Human N	4.83	3.98
Human O	6.83	4.24

[a] Assay using 120 μM Fe(III) citrate.

[b] Assay using monopotassium G6P.

Table IV. Results of Treating Human Hepatic Microsomes with Steapsin

Treatment/Fraction	% of Original P450 Reductase (as cytochrome *c* reductase)	% of Original Cr(VI) Reductase
Pre-Boiled Steapsin:		
pellet	96–97	100
supernatant	3–4	0
Steapsin:		
pellet	4–6	32–34
supernatant	94–96	0
pellet plus supernatant	not tested	250–290

Note: on its own, steapsin does not have any Cr(VI) reductase activity. All studies were conducted with human N microsomes. Microsomes from other humans yielded analogous results.

Table V. Effect of adding purified recombinant human cytochrome b_5 to human N microsomes in a standard NADPH-dependent Cr(VI) reduction assay

Enzyme source*	Cr(VI) Reduction Rate (nmol/min)†
Human N microsomes only	0.272 ± 0.017
Human N microsomes plus human cytochrome b_5	0.598 ± 0.023
cytochrome b_5 only	0.054 ± 0.006

* 130 μg human N microsomal protein ± 10 μg purified recombinant human cytochrome b_5.
† Mean ± S.D., $n = 2$.

Table VI. Comparison of the abilities of purified P450 reductase and human microsomes to reduce cytochrome *c*, Cr(VI), and Fe(III)

Activity Measured	Purified P450 Reductase (PanVera)	Human C Hepatic Microsomes
NADPH: cytochrome *c* reductase activity (nmol/min/mg)	72000.	73.
NADPH:Cr(VI) reductase activity (nmol/min/mg)	69.5	5.00
NADPH:Fe(III) reductase activity (nmol/min/mg)	116.	6.51
ratio of Fe reductase activity to cytochrome *c* reductase activity	0.00161	0.0891
ratio of Cr reductase activity to cytochrome *c* reductase activity	0.000965	0.0685

All values represent the mean of two or more determinations.

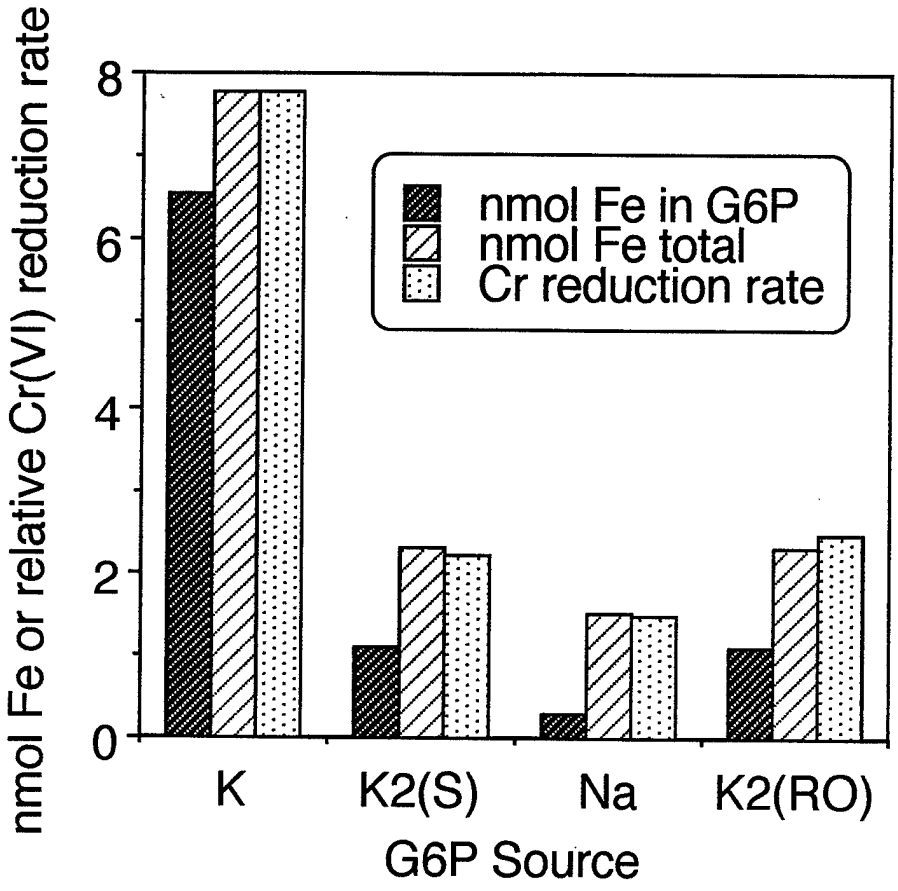


Fig. 1. Correlation between the relative Cr(VI) reduction rate by human hepatic microsomes and the iron content of the G6P salt or the total reaction mix. The salts and suppliers of G6P were as follows: K [monopotassium G6P, Sigma]; Na [monosodium G6P, Sigma]; K2(S) [dipotassium G6P; Sigma]; K2(RO) [dipotassium G6P, Research Organics]. Results shown represent the mean of 3 determinations.

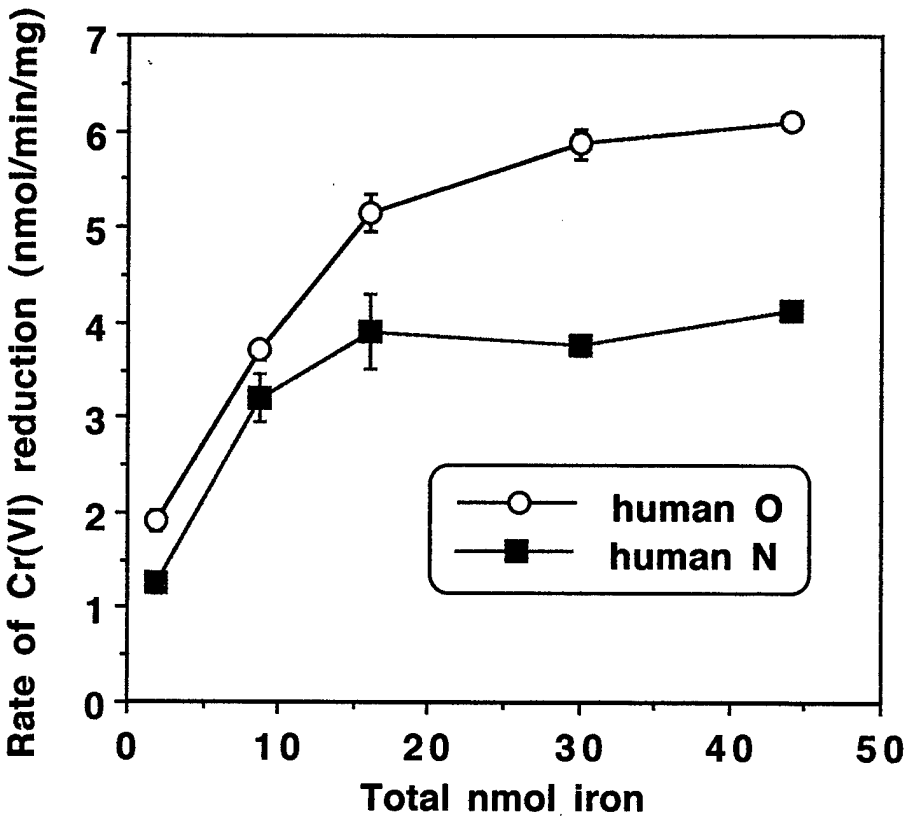


Fig. 2. Effect of adding increasing amounts of $FeCl_3$ to dipotassium G6P (Research Organics) on anaerobic Cr(VI) reduction rates catalyzed by hepatic microsomes from human N (0.260 mg) or human O (0.222 mg). Final total reaction volume was 2.55 ml, and the initial amount of Cr(VI) was 50 nmol. Results shown represent the mean ± S.D., $n = 3$; for points lacking apparent error bars, the bars were smaller than the points as shown.

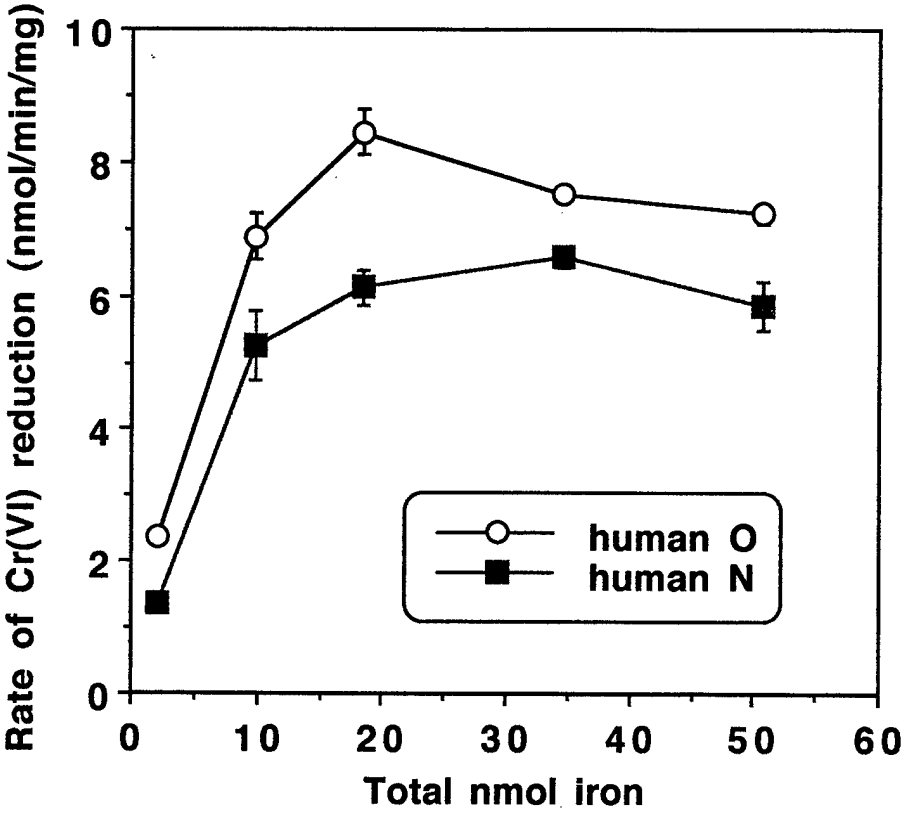


Fig. 3. Effect of adding increasing amounts of $FeSO_4$ to dipotassium G6P (Research Organics) on anaerobic Cr(VI) reduction rates catalyzed by hepatic microsomes from human N (0.260 mg) or human O (0.222 mg). Final total reaction volume was 2.55 ml, and the initial amount of Cr(VI) was 50 nmol. Results shown represent the mean ± S.D., $n = 3$; for points lacking apparent error bars, the bars were smaller than the points as shown.

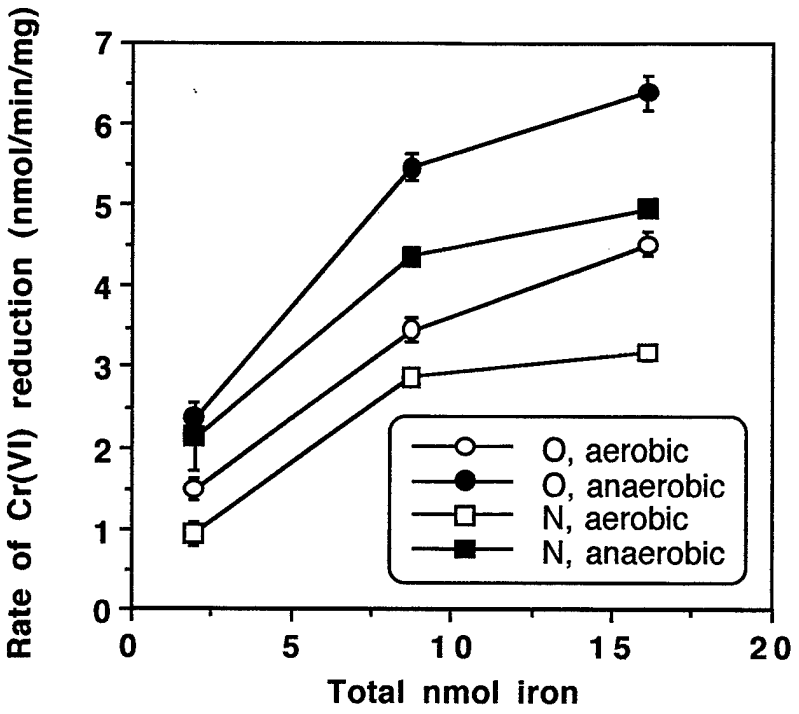


Fig. 4. Effect of adding increasing amounts of $FeCl_3$ to dipotassium G6P (Research Organics) on anaerobic vs. aerobic Cr(VI) reduction rates catalyzed by hepatic microsomes from human N (0.260 mg) or human O (0.222 mg). Final total reaction volume was 2.55 ml, and the initial amount of Cr(VI) was 50 nmol. Results shown represent the mean ± S.D., $n = 3$; for points lacking apparent error bars, the bars were smaller than the points as shown.

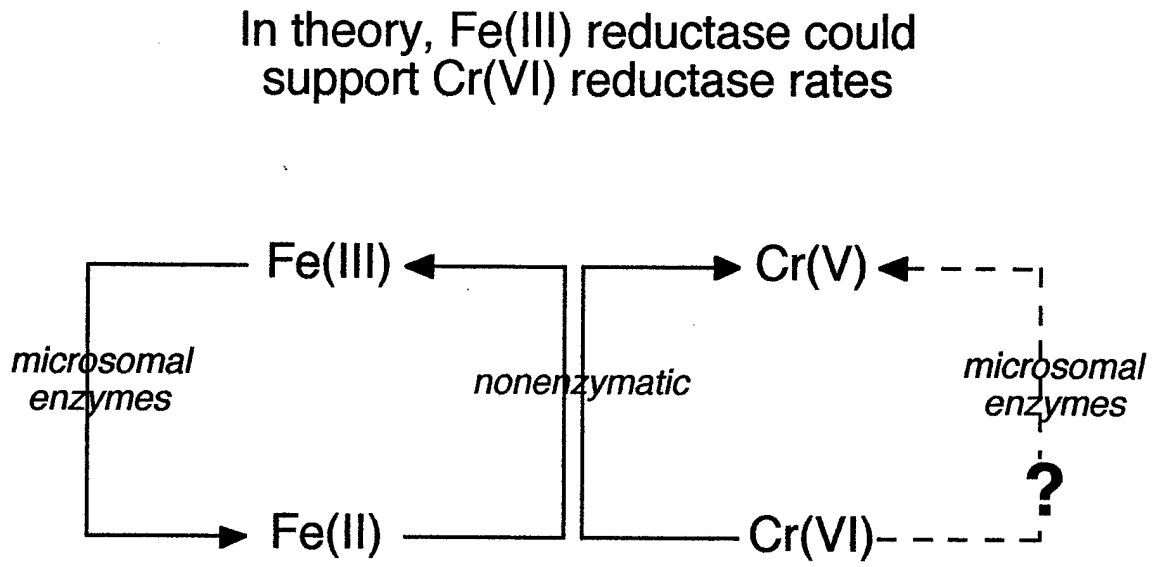
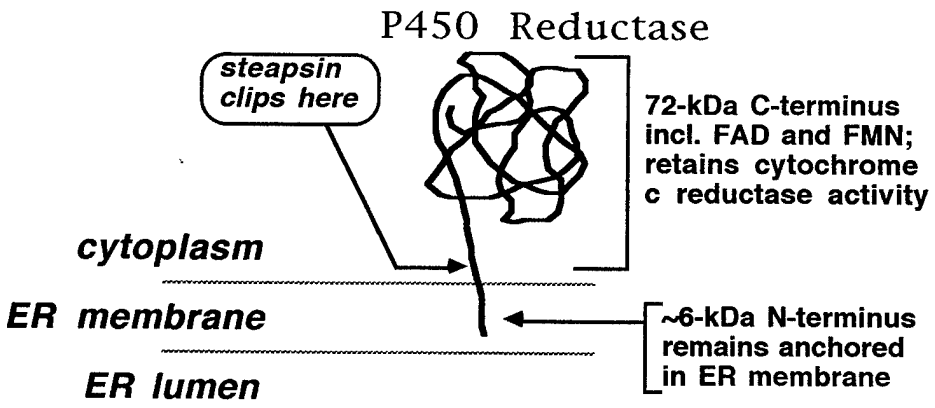


Fig. 5. The rates of microsomal Fe(III) reduction are sufficient to support the observed rates of microsomal Cr(VI) reduction. However, extrapolated "iron-independent" activity comprises 8–30% of the maximal Cr(VI)-reducing activity under high iron conditions, so it is possible that a minority of microsomal Cr(VI) reduction is the result of direct enzymatic reduction.



PROCESS: (1) steapsin treatment of microsomes
(2) ultracentrifuge to separate pellet and supernatant

RESULT: "active" P450 reductase is depleted from microsomal pellet and released into supernatant

Fig. 6. Schematic diagram of how treatment of human microsomes with steapsin releases 72-kDa catalytic fragment of P450 reductase into supernatant.

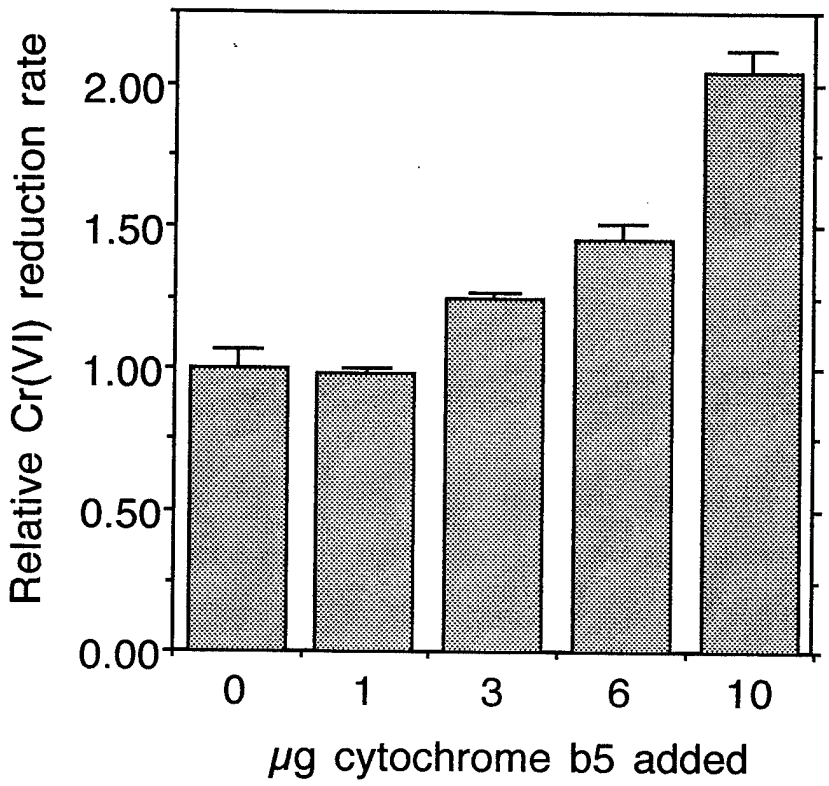


Fig. 7. Effect of adding increasing amounts of purified cytochrome b_5 (PanVera) on anaerobic Cr(VI) reduction rates catalyzed by human N hepatic microsomes (0.13 mg). The final total reaction volume was 2.55 ml, and the initial amount of Cr(VI) was 50 nmol. Results shown represent the mean ± S.D., $n = 2$.

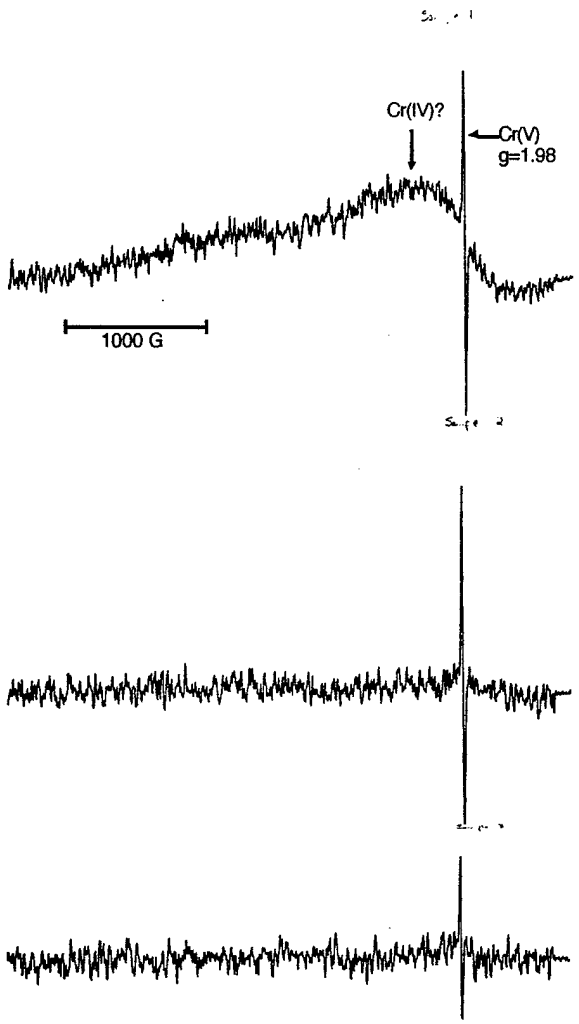
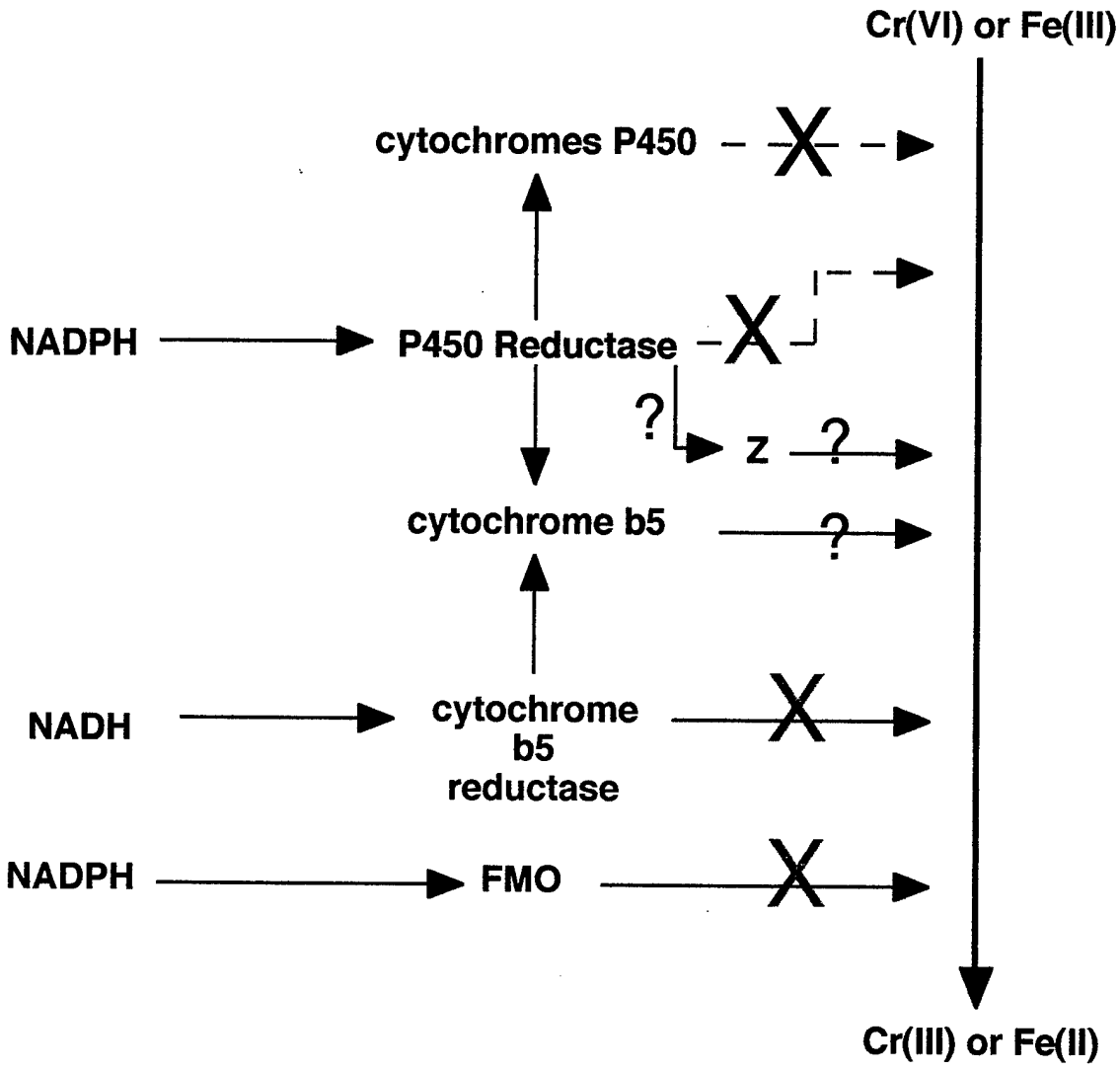


Fig. 8. ESR spectra of Cr(V) formed under various conditions. Top scan: 9 mM Na_2CrO_4 was incubated with 4.6 mM sodium ascorbate in 0.1 M HEPES (pH 7.0) for 80 sec, and then frozen in liquid N_2. Middle scan: 0.8 mM Na_2CrO_4 was incubated under anaerobic conditions with 1 mg human N microsomal protein and the NADPH-generating mix per our standard Cr-reduction conditions [53] for 60 min, and then frozen in liquid N_2. Bottom scan: same as middle except that the microsomes were pre-boiled for 10 min at 100°C before use. ESR instrument settings were: 1.0 G modulation amplitude, 2 x 10^4 receiver gain, 0.128 s time constant. 9.054 GHz microwave frequency, 5 mW microwave power, a sweep width of 4000 G for the entire scan, a 100 K modulation frequency, and a 2 min scan time.



WORKING HYPOTHESIS ON POTENTIAL MECHANISMS OF CHROMIUM OR IRON REDUCTION BY HUMAN MICROSOMES

Fig. 9. Current working hypothesis on the potential mechanisms of chromium or iron reduction by human microsomes. The evidence is clear that cytochromes P450 are not directly involved (black X). Evidence to date suggests that FMO3, the major liver FMO, is also not a significant player (gray X). The possibility remains that other FMO isoforms (e.g. FMO2 in lung) might contribute to Cr(VI) or Fe(III) reduction. P450 reductase is involved in approximately two-thirds of microsomal Cr(VI)-reducing activity, but by itself is a relatively poor reducer of Cr(VI) and Fe(III) (gray X). It appears that P450 reductase acts in concert with other microsomal components, with cytochrome b_5 being a likely possibility. It is also possible that P450 reductase interacts with other as yet unidentified microsomal components. If cytochrome b_5 is involved, then cytochrome b_5 reductase is also likely involved in a NADH-supported process. Recent studies with cytochrome b_5 reductase expressed from a recombinant source suggest that cytochrome b_5 reductase is not a significant direct reducer of Cr(VI) or Fe(III) (gray X), and that its role is likely mediated through interaction with cytochrome b_5.